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# Molecular Analysis of Mutations Induced at the *hisD3052* Allele of Salmonella by Single Chemicals and Complex Mixtures

by David M. DeMarini,[1] Douglas A. Bell,[1] Jessie G. Levine,[2] Melissa L. Shelton,[1] and Amal Abu-Shakra[1]

**More single chemicals and complex environmental mixtures have been evaluated for mutagenicity at the *hisD3052* allele of Salmonella, primarily in strain TA98, than in any other mutation assay. The development of colony probe hybridization procedures and the application of the polymerase chain reaction and direct DNA sequencing has permitted rapid molecular access to this allele. We discuss these techniques and the resulting mutation spectra that have been induced by a variety of environmental mutagens and complex mixtures. A common GC or CG deletion within a hot-spot region of the sequence dominates most of the spectra. In addition to this two-base deletion, we have recovered about 200 other types of mutations within the 72-base target for reversion of the *hisD3052* allele. These include a variety of deletions (as large as 35 bases), duplications (as large as 46 bases), and complex mutations involving base substitutions. The quasipalindromic nature of the target sequence and its potential to form DNA secondary structures and slippage mismatches appear to be an important basis for the mutability of this allele.**

## Environmental Mutagens and Complex Mixtures

As the National Research Council of the National Academy of Sciences has pointed out, people are seldom exposed to single chemicals. Instead, most substances to which people are exposed are mixtures of chemicals (*1*). Thus, people encounter potentially harmful agents primarily through exposure to the air, water, soil, and food. Mutagens present in these media are environmental mutagens of most immediate relevance to human health (*2*). Although people are exposed primarily to mixtures of mutagens, most mutagenicity studies have been performed on single chemicals rather than on complex environmental mixtures.

The first experimental demonstration of the carcinogenicity of a complex mixture (coal tar) was performed in 1915 by Yamagawa and Ichikawa in Japan [reviewed in Nesnow (*3*)]. This has been followed by nearly a century of distinguished research into the carcinogenicity of a variety of complex mixtures such as urban air, diesel engine exhaust, and cigarette smoke. Although a few cytogenetic studies were performed in the late 1950s and 1960s on mammalian cells exposed in culture to cigarette smoke (*4*), the study of the genotoxicity of complex environmental mixtures can be said to have truly begun in 1974 with the report by Kier et al. (*5*) that cigarette smoke condensate (CSC) was mutagenic in Salmonella.

This seminal paper showed that the Salmonella (Ames) mutagenicity assay could be used to screen complex environmental mixtures for mutagenicity. For the first time, a simple and sensitive mutagenicity assay, which had already been evaluated with more chemicals than any other mutagenicity assay, was now available for screening complex mixtures. Perhaps even more importantly, Kier et al. (*5*), following the precedent set in the field of complex mixture carcinogenicity, showed that chemical fractions of a complex mixture could be evaluated for mutagenicity. Just as with the previous carcinogenicity studies, such an evaluation provided insight into which chemical classes were responsible for the mutagenicity of the mixture.

Within just a few years, almost every type of environmental complex mixture imaginable had been evaluated in the Salmonella assay. Organic extracts of air, soil, water, food, etc., were evaluated as well as chemical fractions of these extracts (*2*). It was the use of the Salmonella assay to evaluate these environmental media in the mid- and late 1970s that first alerted us to the presence of mutagens throughout our environment—the extent of which had simply not been known and/or poorly documented and appreciated until that time. During the past 10 years, a

[1]Genetic Toxicology Division, MD-68A, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.
[2]Department of Environmental Science and Engineering, University of North Carolina, Chapel Hill, NC 27599.
Address reprint requests to D. M. DeMarini, U.S. EPA (MD-68A), Research Triangle Park, NC 27711.

large and detailed literature on the mutagenicity of complex mixtures has been produced (*6,7*), providing the basis for mechanistic studies (*8*) and improved risk assessment (*9,10*).

Although other genotoxicity tests in addition to the Salmonella assay have been used to evaluate complex mixtures (*6,7*), the vast majority of the literature on the genotoxicity of complex mixtures has been generated using the Salmonella assay (*2,6,7,11,12*). Recent developments in genome analysis now provide the means by which mutations can be examined at the molecular level (*13*). Thus, we have begun to apply some of these molecular techniques to the analysis of mutations induced in Salmonella by a variety of environmental mutagens and complex mixtures. This paper presents a brief overview of the methods used, the samples evaluated, and some of the major observations and conclusions from our studies of the genotoxicity of complex environmental mixtures.

## Molecular Methods

The Salmonella plate-incorporation mutagenicity assay (*14*) is particularly suited for molecular analysis because of the exquisite selection system and the limited target size (because it is a reverse-mutation assay). In addition, revertants resulting from the plate-incorporation assay are independent in origin and are not sibling revertants because each arises from a single cell that is immobilized and physically isolated from other cells within the top agar. Thus, a mutation spectrum constructed from the mutant sequences of a set of revertants from a single plate can be assumed to be composed of mutations that are independent in origin.

Of all the tester strains, those containing the *hisD3052* allele (especially strains TA1538 and TA98) have been shown to be the most useful in mutagenesis screening programs (*15*) and have been used more than any of the other strains for evaluating complex mixtures (*2,11*). Thus, nearly all of our initial work has focused on the analysis of mutations that revert this allele.

The *hisD3052* allele contains a −1 deletion that was induced by the acridine nitrogen mustard ICR-364-OH (*16,17*). The types of analyses that have been used to characterize revertants of this allele have included *a*) deduction of DNA-base changes based on the amino acid sequence of the histidinol dehydrogenase polypeptide coded by revertants of the *hisD3052* allele (*18*), *b*) cloning and DNA sequencing of revertants (*19,20*), *c*) deduction of DNA-base changes based on colony hybridization of revertants (*21,22*), and *d*) amplification of revertants by the polymerase chain reaction (PCR) and direct DNA sequencing of the amplified fragment (*22,23*).

The use of colony hybridization to identify mutational hot spots, coupled with PCR and direct DNA sequencing to identify the remaining revertants, is the most efficient and rapid method by which to determine the mutations in a large number of *hisD3052* revertants (*21,22*). The greater the number of induced revertants per plate relative to the control value, the greater the probability that the revertants were actually induced by the test agent and are not spontaneous. In the present study, most of the induced revertants that we have analyzed came from plates that had revertant counts that were 7- to 23-fold above the control levels. In addition, we have analyzed spontaneous revertants to compare to the induced spectra.

To purify the revertant clone and to assure that no nonrevertant cells from the background lawn were present, each revertant picked for analysis was first streaked onto minimal medium supplemented with biotin and incubated for 2 days at 37°C. The purified revertants were then subjected to colony probe hybridization to identify a hot spot that consists of a deletion of CG or GC within the sequence CGCGCGCG (*21,22*). Briefly, purified revertants were inoculated into nutrient broth in a 96-well microtiter plate, grown overnight, and replica plated onto brain-heart infusion agar (Difco). After overnight growth, the colonies were transferred to Whatman 541 paper filters and were processed as described (*21,22*). The filters containing the lysed revertants were then placed in plastic petri dishes containing 10 mL of hybridization solution, unlabeled TC-13 probe (added to enhance the hybridization reaction), and $^{32}$P-labeled TC-5 probe (*21,22*). Filters were incubated at 60°C for 2 hr, washed two times for 20 min each in 3 × SSC (0.15 M sodium chloride and 0.015 M sodium sodium citrate, pH 7.0), and dried. The filters were then exposed to Kodak XAR-2 film, and the films were read after overnight exposure.

Because of the common occurrence of the CG or GC deletion (~40–50% spontaneously and even higher among induced revertants), we have routinely screened 400 revertants of TA98 per dose of mutagenic agent, which usually provides for some non-GC or unique revertants that require additional analysis. These were then amplified by PCR and sequenced according to our recently developed method (*23*). Briefly, a single, purified revertant colony was boiled for 10 min in 200 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), centrifuged for 10 min, and 5 μL of the resulting supernatant was used to provide the Salmonella genomic DNA in an asymmetric PCR. The two amplification primers span a 328-bp segment that contains the *hisD3052* mutation approximately in the center. Single-stranded DNA was generated by asymmetric PCR using a 1:100 ratio of the primers and 40 cycles of heating and cooling. We have shown recently that excessive cycling beyond 40 converts the amplified fragment into random-length higher molecular weight fragments, resulting in a dramatic loss of the desired PCR product (*24*).

Excess amplification primers and deoxynucleotides were removed from the PCR mixtures by ultrafiltration using two 2-mL $ddH_2O$ washes in a Centricon-30 microconcentrator (Amicon, Beverly, MA). The amplified DNA was then dried by vacuum centrifugation, resuspended in 10 μL of $dH_20$, and sequenced in a microtiter plate using dITP termination mixes as described (*23*). Because of the high (60%) GC content of Salmonella DNA and the presence of repeating runs of G or GC, compression of the bands on the sequencing gel may occur with standard dNTPs. Thus, dITP termination mixes were used to eliminate this problem.

## Spontaneous Mutation Spectrum

The most surprising observation regarding the spontaneous mutation spectrum at the *hisD3052* allele in strain TA98 (or in TA1538 and TA1978) is the size of the target. Frameshift reversion is generally thought to occur by one of three general mechanisms: *a*) the original sequence is restored exactly to wild type (producing a true revertant), *b*) a second mutation within the gene (intragenic) and near the original mutation occurs to correct the reading frame, or *c*) a second mutation within a suppressor (extragenic) gene (most likely a tRNA gene) occurs that results in the insertion of an amino acid that restores function to the gene product [in this case, histidinol dehydrogenase (*25*)].

Quite unexpectedly, less than 1% of the spontaneous revertants of strain TA98 are true revertants, and the size of the target is at least 72 bases, with the original 1-base deletion approximately in the center (*26,27*). In addition, no extragenic suppressor mutations have been identified. Thus, of the three general mechanisms, 99% of the spontaneous revertants revert by the second mechanism, i.e., intragenic suppressor mutation. Consequently, 99% of the spontaneous revertants of the *hisD3052* allele are not genotypically wild type; instead, they are double mutants at the *hisD* gene.

The likely reason for such a low frequency of true reversion may be due to the large number of alternative reversion pathways that are available within the *hisD3052* target. We have identified about 200 different mutations among revertants of this allele, with at least 50% of these recovered among spontaneous revertants (*26*; DeMarini et al., unpublished observations). Duplications as large as 46 bases and deletions as large as 35 bases have been recovered (*27*). Complex mutations, i.e., those that contain deletions, insertions, and/or base substitutions at or near the same site in a single mutant, occur spontaneously about 3% of the time in TA98, but not at all in TA1538 and TA1978 (*27*). Thus, these mutations appear to result from the error-prone DNA repair system provided by the pKM101 plasmid in TA98. Although deletions occur more frequently spontaneously than duplications in strains TA98 and TA1538, duplications are more dominant in the repair-proficient strain TA1978.

As discussed further in "Mutational Mechanisms," the *hisD3052* allele contains a reversion hot spot that has been recognized by a number of researchers (*18–23*). The reversion event consists of the deletion of a GC or CG within the sequence CGCGCGCG. Thus, seven such mutations can be accommodated within this region. The percentage of spontaneous revertants (+S9) that contain this GC or CG deletion at the hot spot is 47% for TA98, 51% for TA1538, and 15% for TA1978 (Table 1). As shown below, the occurrence of this mutation is frequently increased after mutagen treatment.

## Mutation Spectra Produced by Single Chemicals

The mutations induced by only a relatively small number (~20) of single chemicals have been evaluated at the molecular level at the *hisD3052* allele in Salmonella. Fuscoe et al. (*19*) have reported on revertants induced by the food mutagens PhIP, IQ, and MeIQ, as well as by aflatoxin $B_1$ and benzo[*a*]pyrene. Cebula and Koch (*21*) have presented preliminary reports on revertants induced by adriamycin, daunomycin, aflatoxin $B_1$, benzo[*a*]pyrene, and 2-acetylaminofluorene. Recently, Kupchella and Cebula (*22*) have analyzed at the DNA level the revertants of Isono and Yourno (*18*); these include revertants induced by ICR191, ICR364OH, 2-nitrosofluorene, hycanthone, 4-nitroquinoline-*N*-oxide, and *N*-methyl-*N'*nitro-*N'*nitrosoguanidine. O'Hara and Marnett (*20*) have presented results for β-methoxyacrolein, and we have analyzed revertants induced by 1-nitropyrene (*23*), ellipticine (*28*), the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone [MX (*29*)], and 4-aminobiphenyl (4AB), and the food mutagen Glu-P-1 (*30,31*).

**Table 1. Frequency of hot spot deletions at the *hisD3052* allele in TA98.**

| Chemicals/mixtures | S9 | Frequency, % |
|---|---|---|
| Spontaneous | + | 47 |
| | – | 49 |
| 3-Chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone (MX) | – | 40 |
| 4-Aminobiphenyl | + | 83 |
| Ellipticine | + | 86 |
| 1-Nitropyrene | – | 94 |
| Glu-P-1 | + | 98 |
| Mainstream cigarette smoke condensate | + | 91 |
| Sidestream cigarette smoke condensate | + | 89 |
| Urban air particles | | |
| Unfractionated | + | 89 |
| Base/neutral fraction | + | 90 |
| Municipal waste incinerator particles | | |
| Unfractionated | – | 88 |
| Base/neutral fraction | – | 98 |

It is beyond the purpose and scope of this paper to analyze the results in detail. However, some general observations can be made regarding these studies. Nearly all of these mutagens increase the frequency of the GC or CG deletion hot spot, with the exception of the drinking water mutagen MX (Table 1). Agents that form DNA adducts, such as 4AB and Glu-P-1, produce a number of complex mutations in strain TA98, but not in TA1538 or TA1978 (*30,31*). Thus, it appears that the error-prone DNA repair system conferred by the pKM101 plasmid is necessary to convert these DNA adducts into complex mutations. Although the hot spot deletion dominates the mutation spectra induced by these chemicals, most of the remaining mutations are either complex (in strain TA98) or are similar to the deletions and duplications found in the spontaneous spectrum.

Ellipticine is particularly interesting because, in addition to the hot spot, it also induced two warm spots on either side of the hot spot that were 30 bases apart from each other. When a secondary structure of this region is constructed that contains 66% internal hydrogen bonding, the two warm spots reside opposite each other in looped-out (nonhydrogen-bonded) regions of the hairpin (*28*). Such mutational specificity has not been observed among the other agents we have studied thus far. The drinking

water mutagen MX also may produce an interesting spectrum because only 40% of the MX-induced revertants contain the deletion hot spot, which contrasts with the 83–98% hot spot mutations produced by the other agents we have evaluated (Table 1).

## Mutation Spectra Produced by Complex Mixtures

As discussed previously, many different complex mixtures have been studied for mutagenicity. Of all environmental mutagens, cigarette smoke is perhaps the most important because of its known association with lung cancer and other diseases. Seventeen years after Kier et al. (*5*) showed that CSC was mutagenic in Salmonella, we have identified some of the mutations induced by both mainstream and sidestream CSC (*30*). As with most single chemical mutagens evaluated thus far, about 90% of the CSC-induced revertants of TA98 contain the GC or CG deletion hot spot (Table 1). Although complex mutations make up only 3% of the spontaneous spectrum of TA98, they make up 5 and 8% of the mainstream and sidestream CSC revertants (*30*).

The finding that many of the non-hot spot mutations are complex suggests that they are the result of the error-prone DNA repair system acting on bulky DNA adducts. Such a result is consistent with the types of mutations found in *K-* or *N-ras* genes from lung tumors of mice exposed to polycyclic aromatic hydrocarbons (PAHs) or from lung tumors of human cigarette smokers (*32,33*). The mutations were similar among mouse and human tumors, suggesting that bulky hydrophobic DNA adducts may be responsible for the majority of the mutations observed in the activated human *K-ras* genes from cigarette smokers.

Furthermore, about 50% of the mutations in human *K-ras* and *p53* genes of lung tumors from smokers were G to T transversions, with some others being C to T transitions (*33,34*). Our results with CSC-induced revertants of Salmonella strain TA98 are supportive of these human data in that about 50% of the CSC-induced complex mutations contain G to T base substitutions, and about 50% contain C to T substitutions. This observation is all the more interesting because TA98 is a frameshift mutant that was not designed to detect or necessarily permit the recovery of base substitutions. The recovery of a substantial fraction of base substitutions (all, of course, associated with a frameshift mutation) among the CSC-induced revertants of TA98 suggests that CSC has a strong propensity to induce these base substitutions.

Urban air is another complex mixture that is known to be mutagenic and to which millions of people are exposed (*35*). Thus, we have begun to examine the types of mutations induced by organic extracts of urban air and by chemical fractions of these extracts. Air particles collected during the winter in Boise, Idaho (*36*), were fractionated and evaluated for mutagenicity (*37*) and carcinogenicity (*38*). The results showed that the neutral/base fraction contained 36% of the mass but 81% of the mutagenicity (+S9) of the whole, unfractionated mixture (*37*).

Thus, we generated a mutation spectrum for both the unfractionated mixture and the neutral/base fraction of the air sample. The results showed that about 90% of the revertants in both spectra contained the hot spot deletion (*39*) (Table 1). In addition, about 50% of the remaining mutations were complex, suggesting that large, aromatic compounds in the mixtures formed DNA adducts that were then processed into complex mutations by the error-prone DNA repair system in TA98. The two spectra were similar, which is expected based on the fact that the base/neutral fraction contained 81% of the mutagenic activity of the whole mixture.

We have recently performed a similar study on the unfractionated and base/neutral fraction of the organic extract of particles emitted from a municipal waste incinerator. One major difference between this study and that using urban air is that S9 was used with the urban air samples, but the incinerator samples exhibited potent direct-acting mutagenic activity. Thus, the mutation spectra for the incinerator samples were generated in the absence of S9.

The particles were collected and fractionated as described (*37,40*), and mutation spectra were constructed. The base/neutral fraction of the incinerator particle extract contained 68% of the mass and 79% of the mutagenic activity (*37*). The molecular results were similar to those found for the urban air sample in that about 90% of the revertants of both the unfractionated and base/neutral fraction contained the hot spot mutation (Table 1). Most of the remaining mutations were complex mutations.

To see if particular classes of chemicals were responsible for certain mutations in the spectra, the base/neutral fractions of both the Boise air sample and of the municipal waste incinerator sample were fractionated further by HPLC as described (*41*). Each of the 60 HPLC fractions per HPLC run were evaluated for mutagenic activity in strain TA98 by means of a microsuspension assay (*41*). Several HPLC fractions in each of the mutagenicity profiles (mutagrams) exhibited elevated mutagenic activity, and the revertants produced by these HPLC fractions are currently being analyzed by probe hybridization and PCR/sequencing.

## Mutational Mechanisms

The hot spot deletion dominates both the spontaneous and induced mutation spectra of revertants of the *hisD3052* allele, and it is best explained by the primary DNA sequence at this site. The CGCGCGCG sequence provides more opportunity than does any other sequence at any other site within the target for the occurrence of slipped mispairing within an iterated sequence during replication, resulting in deletions and/or duplications as first described by Streisinger et al. (*42*). Such slipped mispairing is likely to be stabilized by agents that can intercalate and/or form covalent DNA adducts. In addition, the CGCGCGCG region may assume a Z-DNA conformation, which could promote deletion mutations. These mechanisms have been discussed extensively by us and others (*19–23,28*).

The formation of many of the complex mutations in TA98 are compatible with mutation models that involve the misinsertion of a base opposite a DNA adduct followed by slipped mispairing (*28*). Perhaps the most important feature of the *hisD3052* target is the quasipalindromic nature of its sequence, i.e., the fact that it contains many direct and inverted repeated sequences. The ample opportunity for the formation of DNA secondary structures and internal (intrastrand) complementary hydrogen bonding may be the main reason for the high mutability of the *hisD3052* allele by a variety of agents.

As discussed by Ripley (*43*), such structures permit slippage mismatches that can result in frameshifts, and they permit the formation of template-directed mutations. DNA secondary structures and these mechanisms appear to account for a great majority of the mutations recovered among revertants of the *hisD3052* allele (*19–23,28*). Similar mechanisms also explain mutations recovered from a variety of other organisms, including humans (*43*). The recovery of mutations in Salmonella that are similar or identical to those found in other organisms, as well as the apparent similarity in mutational mechanisms across species, offers support for the use of model systems, such as the *hisD3052* allele of Salmonella, for mutation research studies.

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